STUDIES IN ARTIFICIAL PARTHENOGENESIS.[1]

I. Membrane Elevation in the Sea-Urchin Egg.

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Introduction.—The ultimate goal in the study of artificial parthenogenesis is the discovery of the chemical and physical forces which are assumed to cause the initiation of development. The pioneer workers on the problem (Tichomiroff, O. and R. Hertwig, Morgan) were content to regard the phenomenon as the result of a stimulus which arouses the egg from its resting condition. But the present tendency, thanks to the work of Loeb and his school, recognizes that such a theory is merely a cloak for our ignorance, it is little more than a restatement of the problem. For it gives us no information as to the real nature of the assumed stimulus, or in what manner it effects its action. The foremost difficulty in the way of a physical or chemical interpretation is the wide range of chemicals and forces all of which are capable of producing artificial parthenogenesis. None of the theories recently advanced seems capable of explaining all or nearly all of the facts. As a result there has been of late again a return to those vague ideas which challenge attack.

In almost all hypotheses of artificial parthenogenesis, the process, as a result of which a membrane is either pushed out or formed "de novo" around the egg, plays an important rôle. This so-called "membrane formation" is readily observed in the egg of the sea-urchin, and it is in *Arbacia* that I have attempted to study it. I am indebted to Prof. T. H. Morgan for the use of a Columbia table in the Marine Biological Laboratory at Woods Hole, Mass., where I spent the summer of 1912.

The form studied was *Arbacia punctulata* (Gray). The nomenclature of the membranes of the sea-urchin egg is somewhat varied, and it will be necessary to decide on a series of terms, which, for the sake of clearness, shall be exclusively

[1] From the Cornell University Laboratory of Embryology.

used. As it emerges from the ovary the unfertilized egg is already surrounded by a broad band of gelatinous material, the chorion. Directly underneath the chorion lies the peripheral surface of the egg. After fertilization, a membrane is clearly seen beneath the chorion and some little distance from the egg; this is the vitelline (or so-called fertilization membrane). The presence of this membrane in the unfertilized egg has often been disputed. Hertwig[1] was of the opinion that a definite preformed membrane exists. Such a condition seemed to Fol[2] to preclude the penetration of the sperm, a fact which he had for the first time observed. Accordingly, he states that the egg is not surrounded by a membrane, but by a "hyaline layer." Upon fertilization, he observes that this layer becomes lifted from the egg and forms the vitelline membrane. Herbst[3] first showed that this "hyaline layer" of Fol was a true membrane in that it possessed rigidity and was distinct from the underlying cytoplasm. He found on pressing unfertilized eggs under a cover glass, that the protoplasm of the egg flowed out, leaving behind the membrane.[4] Schücking[5] isolated the membrane by cutting the eggs. Finally Kite[6] has been able to dissect away the membrane with the aid of a Barber pipette. No doubt the difficulty in observing the membrane is due to the fact that its refractive index is almost identical with that of sea-water. If the refractive index of sea-water is increased, *e. g.*, by the addition of a protein, the vitelline membrane becomes clearly visible. The observation of this membrane in such liquids of greater refractive index has led several observers to suppose that it is "formed" there. Since membranes "formed" in this way are penetrable to sperm, Loeb[7] has proposed to call them "pseudomembranes."

In as much as the presence of a preformed membrane has been demonstrated by a number of observers, it is incorrect to speak of the "formation" of a vitelline membrane at fertilization.

[1] O. Hertwig, *Morph. Jahrb.*, I., 347 (1875).

[2] H. Fol, *Ann. d. sci. phys. et nat.*, LVIII., 439 (1877); *ib.*, *Mem. d. l. Soc. d. phys. et d'hist. nat. d. Genève*, XXVI. (1879).

[3] C. Herbst, *Biol. Centralbl.*, XIII., 14 (1893).

[4] This fact had already been noted by Hertwig (*loc. cit.*).

[5] Schücking, *Arch. f. d. ges. Physiol.*, XCVII., 68 (1903).

[6] G. L. Kite, *Science*, N. S., XXXVI., 562 (1912).

[7] J. Loeb, *Arch. f. Ent. Mech.*, XXVI., 82 (1908).

Thus, although this phrase is in general use, I have discarded it in preference for the more truly descriptive term "elevation." This is in accord with the German practice.

Loeb claims that the process of normal membrane elevation is too rapid for observation. It is necessary to retard the process by cooling to 3° or 4°, when, on fertilization under these conditions, small rounded elevations, the so-called "bubbles" appear on the egg surface; these flow together and their fused outer boundary becomes the vitelline membrane. A similar effect is observed on treatment with butyric acid. I believe that bubble formation is abnormal, and I shall consider its true cause later.

In the fertilized egg the space between the vitelline membrane and the egg is known as the perivitelline space (Membranraum). Some few minutes after fertilization a hyaline layer appears to be differentiated at the external border of the cytoplasm. This is the hyaline layer (ectoplasmic layer, etc.).

The importance of a study of membrane elevation is twofold. In the first place, it may serve as a device for the prevention of polyspermy in normal fertilization, and is, therefore, of physiologic significance. Secondly, in recent years, this process has played an important part in theories of fertilization and artificial parthenogenesis. Robertson[1] goes so far as to confuse membrane elevation with fertilization, considering the two as synonymous.

Although from time to time various ingenious hypotheses have been advanced to explain membrane elevation, the only theory at all plausible was advanced by Fol[2] who was the first ever to consider the problem. Before discussing Fol's view I shall first mention several other explanations which have been suggested. Schücking[3] thought that the preformed membrane was split by the absorption of water, so that a double membrane was produced on fertilization. Such a process would be difficult to explain physically, and no one has ever attempted to do so. Fischer and Ostwald[4] regard membrane elevation as due to the exit from the egg of a fluid which is given off as a result of coagulation of a portion of the protoplasm. The rapidity with

[1] T. B. Robertson, *Arch. f. Entw. Mech.*, XXXV., 64 (1912).

[2] Fol, *Mem. d. l. Soc. d. phys. et d'hist. nat. d. Geneve*, XXVI. (1879).

[3] Loc. cit.

[4] M. Fischer and Wo. Ostwald, *Arch. f. d. ges. Physiol.*, CVI., 229 (1905).

which the membrane is thrust out is but one of the difficulties which such a theory would have to surmount. Traube[1] considers artificial membrane elevation as the result of a secretion after a return to normal sea-water, but in all but a few cases, such a return to sea-water is not necessary. McClendon[2] is of the opinion that an electro-positive (or acid) colloid secretion is[3] poured out from the egg which reacts with the supposedly electro-negative chorion to produce the vitelline membrane. This view is, of course, not in accord with the evidence cited above in favor of the preëxistence of a vitelline membrane. Elder[4] has recently adopted a conclusion identical with that of McClendon, to whom, however, he makes no acknowledgment. He states that the vitelline membrane does not "form" on eggs which lack a chorion. But these eggs are evidently not in good condition, they are probably overripe, and such eggs have been shown by Fol and Loeb incapable of pushing out a vitelline membrane. Elder also states that if the chorion be removed, membrane elevation does not occur (a fact previously claimed by McClendon) but his method of removal involves keeping the eggs 12–14 hours in artificial sea-water. This, of course, precludes membrane elevation. Such evidence is directly controverted by the fact observed by Lyon[5] that after a most vigorous treatment in the centrifuge normal membrane elevation occurs. In eggs treated in this manner the chorion is always absent.

Fol[6] in discussing membrane elevation in *Asterias* eggs, a process which he states is exactly similar to membrane elevation

[1] J. Traube, *Biochem. Zeitsch.*, XVI., 182 (1909).

[2] J. F. McClendon, BIOL. BULL. XXII., 157 (1912).

[3] As proof that this colloid, which is assumed to lie directly beneath the membrane after fertilization, is electro-positive, McClendon states that the passage of an electric current through fertilized eggs in sea-water causes the membrane to "bulge out" towards the cathode. This effect, however, may very well be due to an attempted migration of the egg in the direction of the anode, towards which electrode, most if not all cells migrate (probably because of the alkalinity of protoplasm). The fact that eggs which have ruptured their membranes do not migrate to the anode (McClendon, *Amer. Journ. Physiol.*, XXVII., 273 (1910)) is probably due to a retarding action of the film of water adjoining the glass slide (see W. B. Hardy and H. W. Harvey, *Proc. Roy. Soc.*, B. LXXXIV., 217 (1911)).

[4] J. C. Elder, *Arch. f. Ent.-Mech.*, XXXV., 145 (1912).

[5] E. P. Lyon, *Arch. f. Ent. Mech.*, XXIII., 151 (1907).

[6] Loc. cit.

in the eggs of the sea-urchins, offers the following explanation. A jelly exists between the surface of the egg and the elevated membrane, for the distance between the egg and membrane always remains uniform on all sides. The pushing out of the membrane is due to the absorption of water by this gel. Herbst[1] in a study of membrane elevation in sea-urchin eggs, supports Fol's interpretation. A similar view is likewise expressed by Krassuskaja and Landau[2] and by R. Hertwig.[3] Nevertheless, the view is sometimes regarded as due to Loeb, who first proposed it in 1908. Let us follow the latter's reasoning a little more closely.

In 1900, he regarded membrane elevation as due to coagulation. In 1905,[4] he thought of it as a secretion. In 1907 he began to consider it as due to liquefaction. He first proves that in general the same types of treatment will produce hæmolysis of the red blood cells and the throwing off of a membrane in *Arbacia*. Then he proceeds to adopt the current theory of hæmolytic action to account for the membrane elevation. Köppe and others had attempted to show that the solution or liquefaction of lipoids was necessary for hæmolysis. Accordingly, Loeb[5] expresses the view that membrane elevation is primarily due to a solution of lipoids at the periphery. This view was also taken up in Loeb's laboratory by v. Knaffl-Lenz,[6] who brought forth evidence that the lipoid in question was lecithin. But the actual force which produced the membrane elevation Loeb believes to be due to the swelling of a colloid. He conceives of this colloid as being given off at the time of fertilization and immediately swelling and thus distending the membrane. This does not accord with his previous statements that the unfertilized egg is naked, but Loeb surmounts this difficulty by assuming an "Oberflächenlamelle" or a differentiated surface layer. In 1909, Loeb[7] is no longer certain that solution of lipoids is necessary,

[1] C. Herbst, loc. cit.

[2] Krassuskaja and Landau, *Biol. Centralbl.*, XXIII., 613 (1903).

[3] R. Hertwig in O. Hertwig's *Handbuch der Entwicklungslehre der Wirbeltiere*, I. (1), p. 484.

[4] Univ. Cal. Publ. Physiology, II., 123 (1905).

[5] Loeb, *Arch. f. d. ges. Physiol.*, CXX., 196 (1908).

[6] v. Knaffl-Lenz, *Arch. f. d. ges. Physiol.*, CXXIII., 279 (1908).

[7] Loeb, "Chem. Entwicklungserregung," p. 248.

but he still clings to the view that the swelling of a colloid is involved. This is the identical view earlier expressed by Fol, Herbst, etc. Because of the similarity between membrane elevation and hæmolysis, Loeb introduces the term lysine to denote any substance of unknown chemical composition which will produce membrane elevation and cytolysis. It is evident that the sperm may contain a lysine.

R. S. Lillie[1] suggests a possible cause of swelling. He assumes that at fertilization the outer surface layer of the egg becomes more permeable to the salts of sea-water. These can now exert no osmotic pressure against it, but since this outer film or membrane must still remain impermeable to the colloids within, these do exert osmotic pressure,[2] and an inflow of water with consequent swelling of some of the peripheral cytoplasm occurs. The cause of such an increase in permeability has only been hinted at. Its actual occurrence Lillie bases on the evidence of McClendon, Lyon and Shackell, and Harvey. But even granted such a change of permeability, might it not just as well be regarded as a result rather than as a cause of membrane elevation? Harvey's[4] view is very much the same. He agrees with Loeb that the membrane elevation is due to the swelling of a colloid, and at the same time brings evidence in favor of Lillie's hypothesis of increased permeability of plasma membrane. But Harvey also believes that the presence of the "membrane substance" is

[1] R. S. Lillie, *Amer. Journ. Physiol.*, XXVII., 301 (1911).

[2] Lillie considers osmotic pressure and "Quellungsdruck" synonymous, but this is obviously improper, for the osmotic pressure of colloids is almost negligible, the "Quellungsdruck," on the other hand, may exert a pressure of over 40 atmospheres.

[3] J. F. McClendon, *Amer. Journ. Physiol.*, XXVII., 240 (1910); Lyon and Shackell, *Science*, N. S., XXXII., 249 (1910); Harvey, *Science*, N. S., XXXII., 565 (1910). The first two papers do not prove increased permeability of the membrane. They only show that fertilized eggs stain more readily than those not fertilized, a phenomenon which may also depend upon an increased rate of adsorption after fertilization, or to a lowering of osmotic pressure, such as Bachmann (*Arch. f. d. ges. Physiol.*, CXLVIII., 141 (1912)) concludes for the egg of *Triton*. McClendon's results are also unsatisfactory. He observed an increased conductivity after fertilization, and from this concluded an increased permeability of the outer layer or membrane. Such an increase in conductivity may well have been caused by a disintegration of some of the eggs. The action of the electric current, or the centrifuging which preceded conductivity determinations, might readily produce disintegration.

[4] E. N. Harvey, *Journ. Exp. Zoöl.*, VIII., 355 (1910).

due to a reaction or reactions brought about by the escape of CO_2 as a result of increased permeability. Unfortunately, however, he does not bring up any evidence in favor of this accessory hypothesis, and it is, indeed, difficult to understand how both reaction and swelling can take place in so short a time.

The appended list is an enumeration of all the various methods by which membrane elevation may be artificially induced in sea-urchin eggs. Sometimes artificial parthenogenesis has been described without any records to show if membrane elevation occurred. Several of these methods are included in the list, where they are followed by a question mark. Most acids only cause membrane elevation after the eggs have been restored to normal sea-water, but in all other cases the process occurs directly.

So-called lipoid solvents:
- Chloroform (Hertwig).
- Toluol, benzol, xylol, oil of cloves, creosote (Herbst).
- Ether, alcohol (Matthews).
- Amylene, phenol (Loeb).

Distilled water (Schücking).
Dilution of sea-water (Schücking).
Isotonic NaCl, KCl (Lillie).
Soap (Loeb).
Saponin, digitalin, solanin (Loeb).
Bile salts (v. Knaffl-Lenz).
Sea-water charged with CO_2 (Delage, Lyon).
Passage of hydrogen and oxygen (Matthews).
Shaking (?) (McClendon).
Heat (34° Loeb) (32° (?) McClendon).
Alkalis (Loeb, Schücking).
KCN (?).
Metallic copper or silver (Herbst).
Electric current (?) (Schücking).
Blood serum (Loeb).
Oöcytin (Robertson).
Higher fatty acids (Loeb).

Membrane elevation only after return to sea-water:
- Lower fatty acids (Loeb),
- Hydroxi-acids (Loeb),
- HNO_3 and HCl (Loeb),

I shall now attempt to prove that every known method of producing membrane elevation results in a lowering of the surface tension of the liquid surrounding the egg. Proceeding in order, the first class of substances noted are those often grouped as

lipoid solvents. The surface tension of some of these substances is listed in Landolt and Börnstein, "Physicalische Chemische Tabellen."

Substance.	Temperature,	Surface Tension (Dynes per Cm.)
Chloroform	20°	26.72
Toluol	17.5°	28.52
Benzol	20°	30.2
Xylol	15.7°	28.97
Ether	20°	16.49
Alcohol	20°	22.03
Amylene[1]	16.5°	17.21
Phenol	36.5°	41.3

It will be noticed that the first four of these substances are practically insoluble in sea-water. Herbst found that chloroform and toluol must be shaken with sea-water to produce the necessary effect.

Distilled Water and Dilution of Sea-water.—The surface tension of distilled water is 75 dynes per centimeter. The addition of inorganic salts always increases the surface tension gradually in proportion to the concentration of each salt present, and, by the work of Valson, Röntgen and Schneider, Whatmough,[2] etc., we should estimate the surface tension of sea-water at approximately 77 dynes per centimeter. Hence diluted sea-water and distilled water have a somewhat lower surface tension than sea-water, and it is to this fact that I attribute their action in causing membrane elevation.

Isotonic Salt Solutions.—Lillie[3] first showed that pure isotonic (*i. e.*, 55M) solutions of sodium salts caused membrane elevation, the order of effectiveness of anions being that of the lyotropic series, $Cl > Br > ClO_3 > NO_3 > CNS > I$. The effect of these salts was inhibited by $CaCl_2$ and $MgCl_2$. It was shown by Whatmough[4] and others that equivalent normal solutions of

[1] The surface tension of amylene is not given in Landolt and Börnstein, so I have taken the value given in Castell-Evans' Physico-Chemical Tables. Creosote is not included in the list as it is a mixture of various substances; most of these, however, have a lower surface tension than water.

[2] Valson, *Ann. de chem. et de phys.* (4), XX., 361 (1870); Röntgen and Schneider, *Wied. Ann.*, XXIX., 165 (1886); Whatmough, *Zeit. f. phys. Chem.*, XXXIX., 129 (1902).

[3] R. S. Lillie, *Amer. Journ. Physiol.*, XXVI., 106 (1910).

[4] *Loc. cit.*

chlorides raise the surface tension of water by approximately equal amounts. Hence a solution of a bivalent salt (such as $MgCl_2$ or $CaCl_2$), which is equal in molecular concentration to a solution of NaCl, gives an increase of surface tension which is about double that produced by the NaCl. Sea-water, consisting as it does of monovalent and bivalent salts, has a higher surface tension than an isotonic solution of sodium chloride (.55M). It also contains sulphates which raise surface tension more than monovalent chlorides. The fact, therefore, that isotonic solutions of NaCl, etc., do cause membrane elevation is exactly in accord with my view. Moreover, the addition of $CaCl_2$ and $MgCl_2$, two bivalent salts, raises the surface tension, and, hence, as might be expected, membrane elevation no longer takes place. Finally, the additional evidence of the correctness of my interpretation is furnished by the order of effectiveness of the ions: $Cl < Br < ClO_3 < NO_3 < CNS < I$. For we know the surface tension increasing powers of the anions to be $Cl > Br > NO_3 > I$.[1]

Soap.—Na oleate greatly lowers the surface tension of water[2] and Loeb[3] finds that when it is added to M/2 NaCl it increases the membrane elevating power of the solution. The fact that as much as 2 per cent. of Na oleate is necessary is probably due to the tendency of the NaCl present to salt it out of solution.

Saponin.—Freundlich[4] determines the surface tension of a solution of saponin as 52 dynes per cm. Probably solanin and digitalin also lower surface tension.

Bile Salts.—These substances are extremely effective in lowering surface tension. For example, Lewis[5] gives the surface tension of a 0.2 per cent. solution of sodium glycocholate as 44.98 dynes per centimeter (at 14°).

Sea-water Charged with CO_2.—The surface tension of gases is zero, hence we should expect them to lower the surface tension of water when dissolved in it. This assumption was proven

[1] Röntgen and Schneider, *Annalen der Physik u. Chem.*, XXIX., 209 (1886); Traube, *Journ. Prakt. Chem.*, CXXXIX., 177 (1885).

[2] Donnan, *Zeits. f. phys. Chem.*, XXXI., 42 (1899).

[3] Loeb, "Chem. Entwicklungserregung," 1909, p. 140.

[4] "Kapillarchemie," p. 56.

[5] Lewis, *Zeit. f. physik. Chem.*, LXXIV., 619 (1910).

correct by Bellati and Lusanna[1] and more recently by Bönicke,[2] who finds that ten volumes per cent. of CO_2 lower the surface tension of water by about one dyne.

Passage of Hydrogen and Oxygen.—Matthews[3] found that if hydrogen be passed through sea-water containing eggs for ten minutes, then oxygen for ten minutes, and once more hydrogen for ten minutes, the eggs thus treated begin to develop. He does not state whether membrane elevation occurs, but we can readily understand that it may well do so, for both hydrogen and oxygen, no doubt, lower the surface tension.[4] As Matthews says he ran the hydrogen through swiftly, the impurities (*e. g.*, SO_3, AsH_3) could not have been completely removed, and probably, for this reason, continued exposure to hydrogen gas was found injurious.

Shaking.—Matthews[5] finds that shaking will cause artificial parthogenesis in starfish eggs, but met with no success in his experiments in *Arbacia.* However, McClendon reports that segmentation occurred after shaking in a vial for five minutes. He does not state if membrane elevation occurred, but from a chance experiment of my own, I believe that it may do so. Vigorous shaking would produce a lowering of surface tension in that it would increase the amount of air absorbed (for effect of gases see above).

Heat (to 32° or Over).—As is well known, a rise of temperature produces a lowering of surface tension in all cases. McClendon states that exposure to 32° for four minutes is sufficient to produce segmentation, but he does not state if membrane elevation occurred.

Alkalis.—Upon the addition of NaOH, KOH, or Na_2CO_3, to sea-water, there is an immediate precipitation of magnesium hydroxide. This fact has never been noted by Loeb, McClendon, or Schücking, all of whom have used alkaline sea-water. It is,

[1] Bellati and Lusanna, *Atti. 1st Venet.* (6), VII. (1889); ref. in *Wied. Beibl.*, XIV., 18 (1889).

[2] K. Bönicke, Dissert., Münster (1905).

[3] A. P. Matthews, *Amer. Journ. Physiol.*, IV., 343, 1901.

[4] The latter gas was investigated by Bönicke, who found that it lowered surface tension.

[5] A. P. Matthews, *Amer. Journ. Physiol.*, VI., 142 (1902).

however, a necessary consequence of the fact that sea-water contains salts of magnesium (which constitute nearly 1/6 of the total salt content). Magnesium salts are always precipitated in the presence of NaOH or KOH, this reaction often being employed in qualitative analysis. As a result of this precipitation, the salts which tend most to raise the surface tension (see p. 350) are precipitated and the surface tension of the sea-water decreases slightly. As might be expected, alkalis are not very powerful agents in producing membrane elevation.

Blood Serum.—Loeb[1] first showed that the body fluid of certain annelids of the family Sipunculidæ was capable of producing membrane elevation even in considerable dilution. As I know nothing of the chemical nature of this fluid, it is useless for me to consider this case. Soon after, he found that mammalian blood serum was also effective.[2] 6.5 volumes of the serum are mixed with one volume of 2.5 M NaCl and the resultant solution is then diluted with 1–9 parts of sea-water. Loeb's results show that the per cent. of eggs which "form a membrane" as a result of treatment with this solution is small, in the majority of cases not a single egg throws off its membrane. In order to obtain a higher percentage of membrane elevation, sensitization must be resorted to. This may be effected either by heating (to 31°–34°) or by the addition to the serum of 3/8 M $BaCl_2$ or $SrCl_2$.

First I shall consider the effects of sensitization, as I believe that this process is in itself capable of producing membrane elevation. The result of heating has already been considered, it having been noted that a lowering of surface tension results. As for the addition of $BaCl_2$ or $SrCl_2$, the result must be obvious. Either one of these salts causes an immediate precipitation of sulphates. Loeb in fact mentions a precipitate of $BaSO_4$ in the case of $BaCl_2$, but fails to do so in the case of $SrCl_2$. Such a precipitation has two effects. (1) The strontium chloride added is precipitated from the solution, which then becomes more dilute. (2) The sulphates of sea-water are replaced by chlorides which are somewhat less effective in elevating the surface tension (see p. 351). Thus sensitization is not such a mysterious

[1] J. Loeb, *Arch. f. d. ges. Physiol.*, CXVIII. (1907).

[2] J. Loeb, *Arch. f. d. ges. Physiol.*, CXXII., 196 (1908); CXXIV., 37 (1908).

process as Moore[1] and Robertson[2] would have us believe when they suppose that it is similar to the process of mordanting before dyeing. An experiment of Moore's seems to show that my explanation is correct. Upon placing eggs in 3/8 $SrCl_2$, and then removing directly into sea-water, sensitization occurs, but if the eggs are first washed in NaCl before being placed in sea-water, the effect of the $SrCl_2$ disappears. Evidently the washing away of the $SrCl_2$ prevents its reaction with sea-water.

The effect of serum alone is very slight. Of nine samples of ox-sera tried by Robertson, only one was effective upon sensitized eggs. I attribute the effect of blood serum to two causes. In the first place, before dilution with sea-water the salt content of the "isotonic" serum is almost wholly NaCl, together with a small amount of KCl. The effect of isotonic NaCl and KCl solutions has already been discussed. Secondly, blood serum contains considerable amounts both of oxygen and of carbon dioxide. The total volume of these gases contained in blood varies greatly. On the average, arterial blood possesses about 20 vols. per cent. of oxygen and 40 vols. per cent. of carbon dioxide, whereas venous blood contains approximately 7 volumes per cent. of oxygen and 50 volumes per cent. of carbon dioxide.[3] Blood serum is richer in carbon dioxide than the blood itself.[4] Thus the serum used by Loeb to produce membrane elevation must contain from 5 to 25 volumes per cent. of carbon dioxide in addition to a lesser amount of oxygen.

No doubt some of this CO_2 is loosely combined, but even in this case it would exert a dissociation pressure which would increase the amount present in solution. Using ox-blood obtained from a slaughter-house, Robertson found a difference in the serum obtained from dark and that obtained from light blood, the former being in all cases more effective. He is at a loss for an explanation, but suggests that the dark blood was obtained from animals deprived of water for some time. Probably the dark color of the more effective blood is associated with the presence of a higher per cent. of CO_2. The action of CO_2

[1] A. R. Moore, Univ. of Cal. Pub. (Physiology), IV., 91 (1912).

[2] T. B. Robertson, *loc. cit.*, p. 345.

[3] See Oppenheimer's "Handbuch der Biochemie," IV. (1).

[4] Hammersten, "Physiological Chemistry," 6th Amer. edition, p. 804.

(and oxygen) in lowering surface tension has already been discussed.

KCN.—Dilute potassium cyanide is practically equivalent to KOH + HCN, as it undergoes hydrolysis. Hence, the statements in regard to alkalis apply also here.

Metallic Copper or Silver.—Herbst[1] found that membrane elevtion was induced by the presence of metallic Cu or Ag. His results were confirmed by Matthews,[2] whose experiments however were performed on starfish eggs. Herbst used silver reduced from silver nitrate, and also a silver coin. In the former case, silver nitrate is probably present and effects a precipitation of chlorides with a consequent dilution of the sea-water and an exchange of chlorides in solution to nitrates. (For a consideration of the effects of dilution, and of the relative effects of various ions, see p. 350.) In the latter case, it is necessary to note that all silver coins are alloys of copper, a metal which Matthews finds to be much more potent than the silver coin itself.[3] The action of copper is due to the fact that this metal is attacked by NaCl in the presence of air. As a result of this reaction copper oxychloride is formed and the solution becomes alkaline.[4] Thus the action of copper is a special case of the action of alkalis, and the metal does not produce an "action at a distance," as Matthews supposes.

Electric Current.—Although an electric current has been used by Schücking and McClendon, neither of these authors states whether membrane elevation occurs. In both cases, copper wires were probably used, so that membrane elevation may have been induced by the action of copper itself, as shown above.

Oöcytin.—This is an hypothetical substance isolated by T. B. Robertson[5] from blood serum and from sperm. Although called a "fertilizing agent" it produces normal membrane elevation on "sensitized" eggs only. On "unsensitized" eggs it produces

[1] C. Herbst, *Mitth. a. d. zool. Station z. Neapel,* XVI., 445 (1904).

[2] A. P. Matthews, *Amer. Journ. Physiol.,* XVIII., 39 (1907).

[3] Metallic silver may itself react for NaCl in the presence of oxygen changes it into AgCl with the simultaneous formation of NaOH. (See Gmelin-Kraut, *Lehrbuch der anorg. Chemie,* II. (2), p. 26.) This reaction probably takes place very slowly, however.

[4] Tilden, *Soc. Chem. Ind.,* V., 84 (1886).

[5] T. B. Robertson, *Arch. f. Ent. Mech.,* XXXV., 64 (1912).

agglutination, and sometimes "blister" formation. Its power of producing membrane elevation is, therefore, chiefly due to the process of "sensitization," the real nature of which has already been considered. The agglutinating effect is due to the presence of free hydrochloric acid. Robertson prepares oöcytin by precipitating blood serum with $BaCl_2$ dissolving the precipitate in n/10 HCl, reprecipitating with acetone, and then redissolving in n/10 HCl. The result of this process can not be other than the isolation of a protein combined with HCl, for we know both $BaCl_2$ and acetone as protein precipitants.[1] And, although Robertson exactly neutralizes his final product with NaOH, he, of course, does not neutralize the combined acid which has no action on the color of the indicator. On dilution, the combined acid is split off. Thus Robertson finds that a dilution of one part in two hundred is necessary in order that the oöcytin become effective. Dilute HCl will produce agglutination in the same way as oöcytin.

Higher Fatty Acids.—Loeb[2] finds that very dilute solutions of heptoic, octoic, nonoic, and caproic acids produce membrane elevation in eggs exposed to them. As Forch[3] and others have shown, the higher fatty acids lower surface tension markedly, even when present in great dilution. The readiness with which a fatty acid lowers surface tension is found to be in direct relation to the number of carbon atoms it contains.

Lower Fatty Acids. Mineral Acids.—There remain to be considered only those cases in which membrane elevation occurs after a return to sea-water. In all of these cases, the solutions in which the eggs are first placed are acidified. All of the acids used lower surface tension and their effectiveness is in direct measure to the readiness with which they do so. The lower fatty acids, formic, acetic, propionic, butyric, and caproic, lower surface tension markedly in solution, and this power increases as we ascend in the series.[4] Similarly, the effectiveness in producing membrane elevation increases with the number of carbon atoms.

[1] Mann ("Physiological Histology," p. 102) finds acetone a precipitant of serum globulin and other proteins.

[2] Loeb, "Chemische Entwicklungserregung," 1909, p. 110.

[3] Forch, *Wied. Ann.*, LXVIII., 801 (1898).

[4] Forch, *loc. cit.*

Dibasic acids, such as oxalic, succinic, and tartaric, do not lower surface tension nearly so much[1] and they have comparatively little effect upon sea-urchin eggs. Hydroxi-acids act in a fashion similar to the dibasic acids. Finally, hydrochloric and nitric acids have been found to be least effective.[2] These acids lower the surface tension but slightly, as has been shown, for example, by Röntgen and Schneider.[3] Loeb[4] could obtain no results with sulphuric acid, and it is interesting to note that this acid does not produce a lowering of surface tension when dilute. The question arises as to the reason that a return to sea-water is necessary in the case of the above mentioned acids. This point will be made clear later.

I have now shown that every known method of producing membrane elevation results in a lowering of surface tension. I also found that a number of hitherto untried substances which lower surface tension, can also be used to produce membrane elevation.

Acetone.—According to von Knaffl-Lenz's view that it is the liquefaction of lecithin which causes membrane elevation (see p. 8, l. 12), acetone should be entirely ineffective, as it is well known for its power of precipitating lecithin. On the contrary, I found acetone a very convenient means of producing membrane elevation. Eggs placed in a solution of 3 or 4 c.c. acetone + 25 c.c. of sea-water push out membranes, and cytolysis follows. A very rapid action results if the eggs are placed in more concentrated solutions of acetone.[5] This can be observed by dropping some eggs into a drop of acetone under the microscope.

Chloretone.[6]—This substance is very effective in producing a lowering of surface tension. I found that a 0.1 per cent. solu-

[1] J. Traube, *Liebig's Annalen*, CCLXV., 27.

[2] In 1905, Loeb (Univ. Cal. Publ. Physiology, II., 113) obtained "practically negative" results on adding these acids to sea-water. In 1909 (*Biochem. Zeitsch.*, XV., 254), he made up his solution in $M/2$ NaCl, which in itself has been shown to cause membrane elevation, and in this way he met with occasional success.

[3] *Loc. cit.*

[4] Loeb, "Chemische Entwicklungserregung," p. 105.

[5] Solutions of acetone in sea-water are not as concentrated as they seem, for the acetone tends to be salted out of solution. (See Bernthsen, "Organische Chemie," p. 170.) The surface tension of acetone at 16.8° is 23.35 dynes per centimeter.

[6] Chloretone is a trade name for tri-chlor tertiary butyl alcohol.

tion lowered the surface tension of water by almost one fourth of its value. As was expected, therefore, membrane elevation was produced when a few crystals of chloretone were added to sea-water containing eggs.

Urethane.—According to an approximate determination, a 0.5 M solution of urethane has a surface tension of about 61.3 dynes per centimeter, a value considerably below that of pure water. Hence all solutions of urethane produce membrane elevation (and cytolysis) whether they be isotonic, hypotonic, or hypertonic. Solutions of urethane in sea-water likewise give results, although this substance is not as effective as chloretone.

Chloral Hydrate acts in similar fashion to chloretone and urethane.

Esters.—All esters possess the property of lowering surface tension.[1] The following esters were used and proved effective in producing membrane elevation. Methyl acetate, ethyl acetate, ethyl butyrate, methyl salicylate.

The substances which lower surface tension are very numerous Not all, however, produce membrane elevation. In general, there are three classes of exceptions.

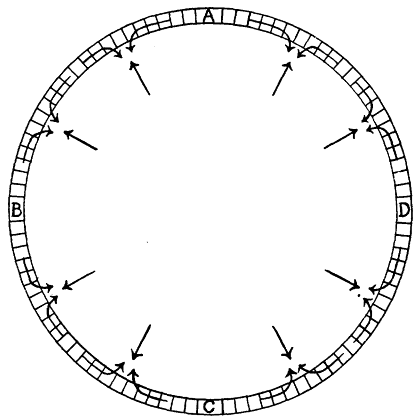
1. *Colloids.*—Many proteins lower surface tension but do not cause membrane elevation.

2. *Protein Coagulants.*—If the solution is strongly coagulative, no sign of membrane elevation will appear. However, if the coagulative action is not very strong, or if the surface tension is quite low, membranes are often pushed out. Under these conditions, a number of little elevations, the so-called "bubbles" or "blisters" are often seen to surround the egg. Loeb and his pupils consider these "blisters" as a preliminary stage in membrane elevation, but they can be observed only when a protein coagulant is present. By adding protein coagulants and at the same time lowering the surface tension, "blister" formation can always be induced. For example, if the eggs are subjected to a solution of acetamide, which contains free acetic acid, they immediately become surrounded with blisters. Similar results are gained with aqueous solutions of picric acid. If the surface tension is low enough, membranes sometimes are formed.

[1] Cf. J. Traube, *Ber. d. deutsch. chem. Gesellsch.*, XVII., 2294 (1884).

3. *Sugar and Glycerin.*—A solution of cane sugar possesses a surface tension very slightly above that of pure water,[1] a glycerin solution has a surface tension below that of sea-water. Thus, both of these solutions have a lower surface tension than sea-water, but if eggs are placed in them, no membrane elevation occurs. The reason for this exception will be considered shortly.

I have shown that membrane elevation depends upon a lowering of surface tension, and I shall now consider the mechanism of the process. As has already been pointed out, the unfertilized egg is surrounded by a membrane, which is probably a gel or semi-gel. In the appended diagram, *ABCD* represents this gel,



whose parts pull on each other by reason of their surface tension. As a result of this pull, the underlying egg contents, and more especially the peripheral portions of the egg, are under pressure. This pressure inward is compensated by a "Quellungsdruck" due to a tendency of the proteins to swell. Upon a diminution of surface tension, the pressure inward is relaxed and the protein or proteins directly beneath the membrane immediately swell, thus pushing out the membrane. If the surface tension remains lowered, the entire egg swells, and cytolysis results.

It was found that not all lowerings of surface tension produced membrane elevation, there being three types of exceptions. The reason for these exceptions can now be explained. Although many colloids lower surface tension, they are unable to diffuse

[1] Forch, *loc. cit.*

into the membrane, and thus lower its surface tension. Moreover, because of their inability to penetrate the membrane, they exert pressure against it, osmotic or otherwise. Secondly, coagulative agents prevent membrane elevation, since they make it impossible for the protein which causes the phenomenon to swell. Probably the main reason that glycerin and sugar solutions do not produce membrane elevation is their action in increasing the viscosity of the gels.[1] When the viscosity of the membrane is augmented, it loses its fluidity and can no longer be pushed out. This hardening effect can be observed directly by placing fertilized eggs with membranes in a sugar solution and examining the result under the microscope.

When acids are used to produce membrane elevation, a return to sea-water is usually necessary. No doubt, the acid by virtue of its coagulative power inhibits membrane elevation. On a return to sea-water the coagulative effect is lost by dilution, but enough acid has probably been adsorbed by the membrane to lower its surface tension sufficiently for membrane elevation.

For the sake of simplicity, one of the factors which must have an influence on membrane elevation has been omitted from the discussion. It has been shown[2] that in the presence of chlorides, bromides or nitrates, gelatine shows a greater tendency to swell than in pure water, but that sulphates, sugar, and glycerin have a retarding effect. If membrane elevation is the result of the swelling of a colloid, and if this colloid behaves as gelatine, the presence of chlorides, bromides, and nitrates would accelerate the process, whereas sugar and glycerin would retard or prevent it. The effect of these substances is probably of secondary importance.

In the course of the argument, evidence has been adduced which may also serve to explain other biological problems. Although the toxicity of distilled water has often been noted, no one has ever offered a satisfactory explanation. Bullot[3] found that the purest water obtainable (redistilled in platinum and quartz) was toxic to the fresh water *Gammarus*. It is believed that this

[1] Leick, *Drude's Annalen*, XIV., 139 (1904).

[2] See Freundlich, "Kapillarchemie," p. 512.

[3] G. Bullot, Univ. Cal. Pub. Physiol., I., 199 (1904).

toxicity is due to the lower surface tension possessed by pure water (see p. 350). The experiments of R. Lillie have often been adduced as evidence in favor of the "theory of antagonism." Lillie's results find a most suitable explanation when the surface tension of the solutions is considered (see p. 351). A recent paper by Lillie[1] seeks to show an antagonism between salts and anæsthetics in their action on starfish and sea-urchin eggs. This would seem to contradict the results gained in this paper. All that Lillie shows, however, is that various anæsthetics, such as ether, chloroform, etc., exert a slight protective influence on eggs partially cytolyzed with isotonic NaCl solution. This protective action is probably due to the effects of the anæsthetics upon the bacteria which are always found to infest cytolyzing eggs. When these are killed the egg is able to live longer. Gorham and Tower[2] showed that sea-urchin eggs could be kept in healthy condition in sterile sea-water for 11 days or longer, whereas they soon disintegrate if exposed to bacteria.

Summary.

1. All known methods of producing an elevation (formation) of the vitelline membrane in the egg of the sea-urchin result in a lowering of surface tension.

2. The following substances, all of which lower surface tension, were also found effective in producing membrane elevation: acetone, chloretone, urethane, chloral hydrate, methyl acetate, ethyl butyrate, methyl salicylate, acetamide, picric acid.

3. A simple physical explanation of the process is given, which is based on Fol's original interpretation.

I desire to express my sincere thanks to Prof. B. F. Kingsbury of Cornell University for helpful advice in the preparation of this paper. Thanks are also due to Prof. W. D. Bancroft, who has been consulted on several questions of a chemical nature.

[1] R. S. Lillie, *Amer. Journ. Physiol.*, XXX., 1 (1912).

[2] F. P. Gorham and R. W. Tower, *Amer. Journ. Physiol.*, VIII., 175 (1902).